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Ikaros Sets Thresholds for T Cell Activation and Regulates Chromosome Propagation

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Summary

T cell activation involves the sustained accumulation of T cell receptor (TCR) and IL-2 receptor (IL-2R) mediated signaling events that promote cell cycle entry and progression. The Ikaros family of nuclear factors regulate this process by providing thresholds overcome by receptor signaling. T cells with reduced levels of Ikaros activity require fewer TCR engagement events for activation, exhibit a greater proliferative response to IL-2, and are less sensitive to inhibitors of TCR and IL-2R signaling. Upon T cell activation, Ikaros proteins localize in a higher-order chromatin structure where they colocalize with components of the DNA replication machinery. Proliferating T cells with reduced Ikaros activity display chromosome abnormalities. We propose that participation of Ikaros in higher-order chromatin structures controls cell cycle transitions and restricts DNA replication.

Introduction

T cell development involves a tightly regulated succession of proliferation and differentiation steps that generate the appropriate number, type and specificities of functional mature T cells. During thymocyte differentiation, the pre-T cell receptor complex, expressed on double negative ($CD4^-/CD8^-$) precursors, mediates their proliferative expansion and differentiation to the double positive stage ($CD4^+/CD8^+$). Signaling via the T cell receptor (TCR) complex expressed on late double positive ($CD4^+/CD8^+$) thymocytes has a variety of nonproliferative outcomes. High-affinity interactions between T cell antigen receptors and self-antigens lead to the apoptotic elimination of double positive cells, whereas engagement of receptors with low affinity for self causes prolongation of their life span and transition to a mature state (reviewed in Janeway, 1994). Mature naive T cells follow a similar sequence of proliferation and differentiation events, with the cellular outcome of antigenic stimulation dependent on the combination of antigen receptor and coreceptors involved. Engagement of antigen receptors on mature peripheral T cells can lead to activation or anergy, a state that is determined by the number of TCRs involved, the duration of their engagement and whether costimulatory or inhibitory receptors are also triggered (Valitutti and Lanzavecchia, 1997; Iezzi et al., 1998).

TCR, costimulatory and cytokine receptor signaling cascades ultimately target nuclear effectors which are responsible for changing the pattern of gene expression and the cell cycle status and can influence the state of differentiation of the responding cell (Crabtree and Clipstone, 1994; Ihle and Kerr, 1995; Wingren et al., 1995; Cantrell, 1996; Johnston et al., 1996; Rao et al., 1997). TCR-mediated induction of IL-2 and IL-2 receptor (IL-2R) expression are early events in T cell activation required for G1 progression and entry into S phase. Combinatorial signaling via the TCR and IL-2 R downregulates the cell cycle inhibitor p27 and activates the cdk4/cdk6 cyclin D and cdk2/cyclin E complexes that promote G1 progression and entry into S phase. Although a number of nuclear factors have been identified that control cell cycle transitions in a variety of cell types (Nourse et al., 1994; Elledge, 1996), in quiescent T cells, TCR and coreceptor signaling cascades may target additional lymphoid-specific nuclear effectors for entry into the cell cycle. Identifying such lymphoid-restricted cell cycle regulators is critical in delineating the molecular events that underlie stages of T cell differentiation and function as well as the molecular mechanisms that give rise to a host of lymphoid defects such as lymphoproliferative disorders, autoimmunities and immunodeficiencies.

The *Ikaros* gene encodes a family of hemopoietic-specific transcription factors that are essential for commitment of early hemopoietic progenitors to the B and T lymphoid lineages (Georgopoulos et al., 1994; Hahm et al., 1994; Wang et al., 1996). Lack of Ikaros proteins in the hemopoietic system causes an early and complete arrest in the production of fetal and postnatal B lymphocytes and natural killer cells. T cell differentiation is also blocked at the fetal stage; however, after birth a small number of T cell precursors appears in the thymus which undergo aberrant differentiation skewed toward the CD4 lineage (Wang et al., 1996). *Ikaros* also plays a critical role in T cell homeostasis. Lack of or a substantial reduction in the levels of Ikaros DNA-binding activity in immature thymocytes, as engineered in mice by a null or a dominant-negative mutation in *Ikaros*, causes their clonal expansion and rapid transformation to a neoplastic state (Winandy et al., 1995; Wang et al., 1996).

Given the dramatic effects that reduction of Ikaros has on T cell homeostasis, we tested its role in T cell activation. Here we report that, upon T cell activation, Ikaros proteins enter into a higher-order nuclear structure where they colocalize with components of the DNA replication machinery. We show that T cells with reduced levels of Ikaros activity exhibit facilitated entry into the cell cycle in response to TCR and IL-2R engagement and are less sensitive to inhibitors of these signaling pathways. Finally, analysis of primary cycling T cells with reduced Ikaros activity revealed aberrant chromosome structures implicating Ikaros as a regulator of chromosome propagation.

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Results

Reduction in Ikaros Activity Causes a Reduction in T Cell Activation Thresholds

Given the hyperproliferative phenotypes manifested in vivo among T cells deficient for Ikaros activity (Winandy et al., 1995; Wang et al., 1996), we examined how T cells with reduced Ikaros activity respond to T cell activation relative to wild-type. For this study, splenic T cells heterozygous for either an inactivating (null$^{+/-}$) or a dominant-negative (DN$^{+/-}$) mutation in *Ikaros* were used. Based on FACS analyses of thymic and splenic populations, these do not appear to develop aberrantly, unlike null$^{-/-}$ T cells. Ikaros null$^{+/-}$ T cells display a 50% reduction in *Ikaros* mRNA (Figure 2A, Ik) and protein (data not shown) relative to wild-type. In Ikaros DN$^{+/-}$ T cells, the level of Ikaros DNA-binding complexes would be further reduced since proteins generated by the mutant allelle lack a DNA-binding domain but can dimerize with the DNA binding isoforms generated by the wild-type allelle and, in principle, interfere with their activity (Sun et al., 1996).

T cells were activated with increasing concentrations of a plate-bound monoclonal antibody against the CD3ϵ invariant component of the TCR complex and the amount of DNA synthesis was determined as a measure of activation and progression through the cell cycle. Since the number of TCR molecules per cell was similar between wild-type and Ikaros-mutant T cells (Figure 1A), ligand (i.e., antibody concentration) should be proportional to signaling in both cell types. However, T cells with reduced levels of Ikaros activity proliferated better than wild-type over a range of antibody concentrations (Figure 1B). In particular, a limiting number of TCR-complex engagement events not sufficient to promote significant activation in the wild-type T cell population caused the robust proliferation of T cells with reduced levels of Ikaros activity (Figure 1B, 0.625 to 20 μg/ml of anti-CD3).

T cell activation causes the production of cytokines required to sustain proliferation, such as IL-2 and IL-4. Cell density is, therefore, a critical parameter in lymphocyte activation assays, where growth factors serve as both autocrine and paracrine stimulators of proliferation. T cells with reduced levels of Ikaros proliferated at low cell densities at which wild-type T cells did not show any significant response (Figure 1A, 5×10^4–10^5 cells/well). Moreover, null$^{+/-}$ and DN$^{+/-}$ T cells differed in prolifertive responses (Figure 1B, 0.625–2.5 μg/ml of anti-CD3ϵ at 5×10^4–10^5 cells/well). At low cell density and under limiting TCR engagement, the proliferative response of Ikaros DN$^{+/-}$ T cells was 3- to 6-fold greater than the Ikaros null$^{+/-}$ T cells, which proliferated 2- to 7-fold better than wild-type T cells (Figure 1B, 0.625–5 μg/ml of anti-CD3ϵ and 5×10^4 cells/well).

We, therefore, conclude that the number of TCR signaling events required to drive a quiescent T cell into the cell cycle depends on the level of Ikaros DNA-binding proteins present in the nucleus. Ikaros DN$^{+/-}$ T cells are less signal-dependent than Ikaros null$^{+/-}$ T cells, which in turn are less signal-dependent than wild-type. These data suggest that Ikaros proteins act as a counting mechanism which monitors TCR signaling events and controls T cell activation.

Ikaros-Mutant T Cells Exhibit Accelerated Entry into S Phase

The cell cycle distribution of wild-type and Ikaros-mutant T cells was examined at different time points after TCR engagement. For this study, T cells were plated under nonlimiting activation conditions (i.e., at high cell density and with a high concentration of anti-CD3ϵ), and the DNA content was monitored from 24–60 hr (Figure 1C). At 24 hr, the majority (93.8%) of wild-type T cells had not yet entered S phase and the amount of DNA synthesis was minimal (Figure 1C). In sharp contrast, a significant number (17% and 17.8%) of T cells heterozygous for either of the *Ikaros* mutations were in the replicative phase of the cell cycle (Figure 1C). However, after 36–60 hr of stimulation, a similar proportion (41.4–51.3%) of wild-type and Ikaros-mutant T cells were in S phase (Figure 1C).

Reducing Ikaros DNA-binding activity not only facilitates the G0 to G1 transition under fewer TCR engagements, it also accelerates the progression from G1 into S normally mediated by IL-2R signaling (Bierer et al., 1990). Memory T cells also display a facilitated entry into the cell cycle in response to antigenic stimulation (Pihlgren et al., 1996). To determine whether T cells with reduced Ikaros activity have aquired a memory cell phenotype, presumably by undergoing aberrant differentiation, we tested expression of the memory T cell differentiation markers CD45RA and CD44. No difference in the percentage of cells expressing these markers was detected between wild-type and Ikaros-mutant T cells (data not shown).

Activated Ikaros-Mutant T Cells Hyperrespond to IL-2

Wild-type T cells are more signal-dependent than Ikaros-mutant T cells and activate efficiently only at a higher cell density, where either supporting factors or cell-cell interactions suffice to overcome activation thresholds and allow entry into S phase. To determine whether Ikaros-mutant T cells provided more growth- and differentiation-supporting cytokines, the intracellular levels of IL-2, IL-4 and IFN-γ were examined after activation. Five hours after TCR stimulation, no significant difference in the intracellular levels of IL-2 or IL-4 or in the number of IL-2$-$ (Figure 1D) and IL-4$-$ (data not shown) producing cells was detected in Ikaros-mutant relative to wild-type T cells. An increase in the number of IFN-γ-producing cells was detected among activated Ikaros-mutant T cells (Figure 1D). Since IFN-γ is not a growth but rather a T cell differentiation factor, it is unlikely to account for the difference in T cell proliferation.

Given that IL-2 is a dominant autocrine growth factor in T cell activation (Smith, 1988), the TCR-mediated proliferation of Ikaros-mutant and wild-type T cells was examined with and without IL-2 added in the culture medium. After 24 hr of TCR stimulation and at low cell density, there was a 6-fold difference in DNA synthesis between Ikaros-mutant and wild-type T cells (Figure 1E, 2×10^4 cells). However, when IL-2 was added to the low density culture (2×10^4 cells), the proliferative difference between mutant and wild-type T cells increased to 70- to 80-fold, with the majority of wild-type cells failing to proliferate (Figure 1E). At higher cell density (5×10^4

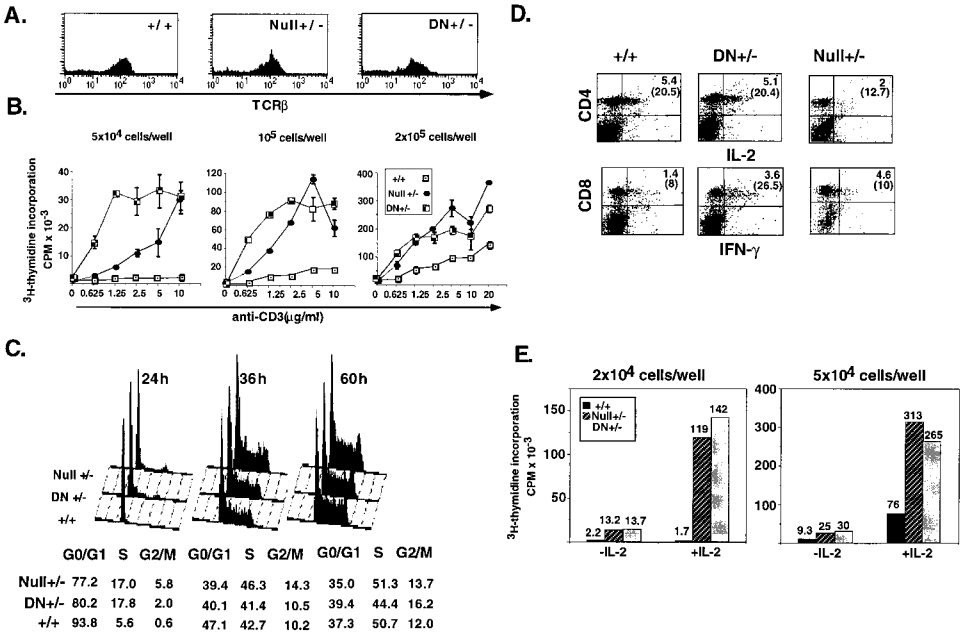


Figure 1. Lower Thresholds of Activation, Accelerated Entry into S, and Hyperresponsiveness to IL-2 in T Cells with Reduced Levels of Ikaros

(A) Wild-type (+/+), Ikaros Null$^{+/-}$, and Ikaros DN$^{+/-}$ T cells were stained for expression of TCR.

(B) Wild-type (+/+), Ikaros Null$^{+/-}$, and DN$^{+/-}$ splenocytes were activated with decreasing amounts of anti-CD3ϵ antibody. The average ^{3}H-thymidine incorporation was measured after 48 hr of culture. Counts (CPM) due to autoproliferation were for 5×10^4 cells: 720 (+/+), 525 (Null$^{+/-}$), 950 (DN$^{+/-}$), for 1×10^5 cells: 1062 (+/+), 730 (Null$^{+/-}$), and 1917 (DN$^{+/-}$), for 2×10^5: 2768 (+/+), 2419 (Null$^{+/-}$), and 6438 (DN$^{+/-}$). The same percentage of T cells was present in all three splenocyte populations. Similar results were obtained with purified T cells (data not shown). The graph shown is representative of three separate experiments. Each point plotted represents the average of triplicate wells, and the error bars represent the standard deviation.

(C) Purified splenic T cells were cultured in the presence of 20 μg/ml plate-bound anti-CD3ϵ and DNA content was determined by propidium iodide (PI) staining after 24, 36, and 60 hr. Histograms display PI intensity. Shown below are the percentages of cells in G0/G1, S, or G2/M.

(D) Two-color FACS analysis showing staining of activated wild-type, Ikaros Null$^{+/-}$ and DN$^{+/-}$ splenocytes for CD4, CD8 and intracellular IL-2 and IFN-γ. Percentage of positive cells is shown in the upper right quadrant. In parentheses is the percentage of CD4 or CD8 cells in the culture positive for IL-2 or IFN-γ, respectively.

(E) Purified splenic T cells were cultured for 17 hr with 2.5 units/ml of IL-2 and pulsed with ^{3}H-thymidine. ^{3}H-thymidine incorporation was estimated as the average of triplicate samples.

cells), addition of IL-2 caused only a 3- to 4-fold difference in proliferation between Ikaros-mutant and wild-type T cells (Figure 1E), indicating that, in the presence of sufficient cell density-dependent signals, both mutant and wild-type cells had effectively entered S phase. In summary, the TCR-mediated hyperresponsiveness of Ikaros-mutant T cells cannot be attributed to an increased production of IL-2 but is possibly due to synergy with a hyperactive IL-2R signaling pathway.

Normal Expression of Some Signaling and Cell Cycle Regulators in Ikaros-Mutant T Cells

The facilitated proliferation of Ikaros-mutant T cells in response to IL-2 implies a deregulation in either IL-2R expression or in a downstream signaling pathway. In both Ikaros-mutant and wild-type T cells, IL-2R was not expressed in the resting state (Figure 2A), and the kinetics of induction upon activation were similar (detected at the cell surface 6 hr after activation, data not shown). Therefore, in Ikaros-mutant T cells, IL-2R expression is not altered, although it is clearly upregulated as they transform to a malignant state (Winandy et al., 1995).

We next examined expression of several regulators of cell cycle progression. To maximize the possibility of observing differences between wild-type and Ikaros-mutant T cells we used DN$^{+/-}$ T cells, as they exhibit the most pronounced hyperproliferative phenotype. The cell cycle inhibitor p27 is downregulated at the protein and mRNA levels to allow G1 progression and entry into S phase (Nourse et al., 1994; Kwon et al., 1997). Protein levels of p27 were comparably high in resting and declined in activated DN$^{+/-}$ and wild-type T cells (Figure 2B). This downregulation was also observed at the mRNA level (Figure 2A). In contrast to p27, the cell cycle inhibitors p19 and p21 are upregulated upon T cell activation (Nourse et al., 1994), presumably to regulate the proliferative response. Protein levels of both of these inhibitors increased similarly in DN$^{+/-}$ and wild-type T cells (Figure 2B). Finally, expression of cyclins D, E, A and cdk2, which promote transition through G1 to S, was comparably low in resting cells and similarly upregulated in DN$^{+/-}$ and wild-type T cells (Figure 2B). Thus, the expression of several key factors for T cell cycle entry and progression—IL-2, IL-2R, p27, p21, p19, cdk2 and cyclins D, E and A—is normal in DN$^{+/-}$ T cells. However, it is still possible that expression of some other cell cycle regulator is affected in these cells.

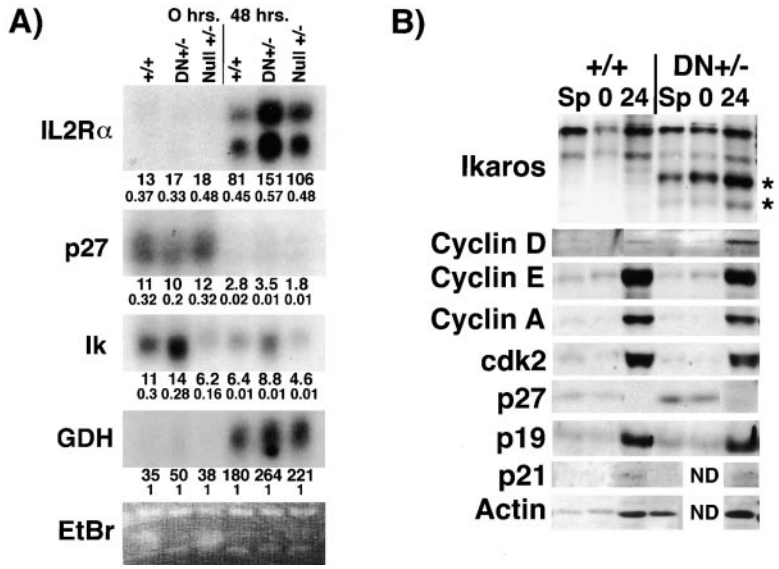


Figure 2. Expression of Cell Cycle Regulators in Ikaros-Mutant T Cells

(A) Northern blot analysis of IL-2Rα chain, p27 and Ikaros mRNA levels in resting T cells versus activated T cells (for 48 hr). The housekeeping gene GAPDH was used as a control. Counts in each band are shown below each panel. The first row of numbers gives the absolute counts (×1,000). The second row gives a relative value obtained by dividing the counts of each band by the counts obtained for GAPDH in the same lane. Note induction of GAPDH in activated compared to resting T cells. Thus, comparison of relative expression of Ikaros, IL-2Rα and p27 in resting versus activated cells is not valid. Ethidium bromide-stained gel displayed in the lowest panel (EtBr) reveals approximately equal RNA loading per lane and allows for comparison of absolute counts in resting versus activated T cells.

(B) Western blot analysis of extracts from resting +/+ and $DN^{+/-}$ splenocytes (Sp), resting T cells (0) and T cells activated for 24 hr (24). Whole cell extracts from 10^6 cells were loaded per lane. Antibodies used are shown on the left. Asterisks indicate dominant-negative Ikaros isoforms generated by the mutant locus. ND = not done.

Reduced expression of receptors or factors which negatively regulate signaling (i.e., CTLA-4 or SHP-1) can also result in TCR-mediated hyperproliferation (Tivol et al., 1995; Marengère et al., 1996; Plas et al., 1996). However, expression of these negative effectors was found to be normal in Ikaros-mutant T cells (data not shown).

Proliferation of Ikaros-Mutant T Cells Is Less Sensitive to Signaling Inhibitors

One of the earliest events post receptor engagement is activation of the src-family protein tyrosine kinases (PTKs) fyn and lck (reviewed in Weiss, 1993). Tyrosine phosphorylation levels in resting and activated T cells were comparable in wild-type and Ikaros-mutants (data not shown), indicating no major differences in PTK activity.

PTKs are selectively inhibited by the pharmacological agent PP1 (Hanke et al., 1996) (Figure 3A). With increasing concentrations of PP1, wild-type T cells stimulated with anti-CD3ϵ showed a dose-dependent decrease in proliferative capacity (Figure 3B). In contrast, Ikaros-mutant T cells displayed enhanced proliferation in the presence of up to 100 ng/ml PP1. However, at a higher concentration (200 ng/ml) of PP1, a decrease in the proliferative capacity of Ikaros-mutant T cells was also observed.

Downstream of the src-PTKs are three major pathways whose signals converge in the nucleus, leading to changes in gene expression and cell cycle progression (Cantrell, 1996). One includes protein kinase C (PKC), Ras and the MAP kinase cascade (Figure 3A). Inhibitors of proximal and distal components in this pathway were examined for their effects on the proliferation of Ikaros-mutant T cells. Staurosporine (PKC inhibitor) (Tamaoki et al., 1986), L739,749 (farnesylation inhibitor of Ras [Weiss, 1993]), and PD98059 (MEK-1 inhibitor) [Migone et al., 1998]) were tested. As observed with PP1, proliferation of Ikaros-mutant T cells was virtually unaffected by the addition of the Ras inhibitor L739,749, except for an increase in proliferative capacity at low inhibitor concentrations (Figure 3B). In addition, mutant cells were less sensitive to the inhibitors of PKC (staurosporine) and MEK-1 (PD98059) (Figure 3B). In fact, Ikaros $null^{+/-}$ and $DN^{+/-}$ T cells, showed progressively decreasing sensitivity to PD98059 at concentrations which had profound effects on the proliferation of wild-type T cells (Figure 3B, at 50 μM, 20% activity for wild-type versus 70% $null^{+/-}$ and 97% activity for $DN^{+/-}$).

A second pathway downstream of the src-PTKs involves phosphatidylinositol 3-kinase (PI 3-kinase) (Figure 3A). PI 3-kinase can be selectively inhibited by the pharmacological agent wortmannin (Powis et al., 1994). Ikaros-mutant T cells were dramatically less sensitive to inhibition of proliferation by wortmannin than wild-type T cells. At 100 nm, wild-type T cells showed less than 10% maximum proliferative capacity, whereas Ikaros $null^{+/-}$ and $DN^{+/-}$ T cells showed 83% and 63%, respectively (Figure 3B).

A third pathway downstream of the PTKs, which is inhibited by cyclosporin A, involves phospholipase Cγ1 (PLCγ1), an increase in intracellular Ca^{2+} and activation of the protein phosphatase calcineurin (Hanke et al., 1996) (Figure 3A). At low concentrations (1 ng/ml), cyclosporin A acted as a potent inhibitor of proliferation for wild-type T cells but had only slight effects on Ikaros-mutant T cells (Figure 3B). At higher concentrations, however, it was effective in inhibiting the proliferation of Ikaros-mutant T cells.

Signaling through IL-2R mediates activation of mTOR and p70 S6 kinase through the MAP kinase pathway and is required for the G1 to S transition (Burnett et al., 1998). Rapamycin is a specific inhibitor of mTOR,

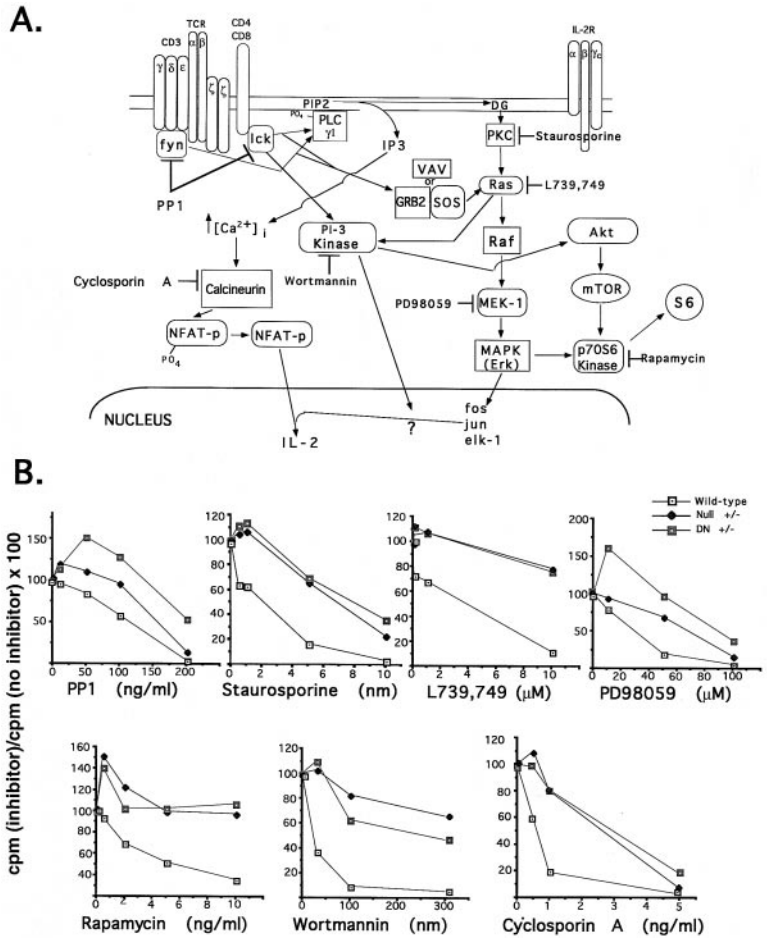


Figure 3. T Cells with Reduced Levels of Ikaros Are Less Sensitive to Signaling Inhibitors

(A) Diagram depicts molecular components of the PKC, PI-3 kinase and calcineurin signaling pathways which lie downstream of TCR complex and fyn/lck. The PKC pathway is also operational downstream of IL-2R. The points of action of inhibitors used in this study are indicated.

(B) Purified splenic T cells from +/+, Null$^{+/-}$, and DN$^{+/-}$ mice were plated in the presence of 20 μg/ml plate-bound anti-CD3ε and inhibitors. After 44 hr of culture, cells were pulsed with ^{3}H-thymidine and the ratio of the average incorporation per well in the presence versus absence of inhibitor (×100), is plotted against inhibitor concentration. Background proliferation of cells plated in the presence of hamster IgG was similar for all three genotypes.

thus preventing phosphorylation of p70 S6 kinase and blocking IL-2R signaling (Kuo et al., 1992; Price et al., 1992; Kwon et al., 1997). Proliferation of Ikaros-mutant T cells was unaffected by the presence of rapamycin over a range of concentrations effective in blocking proliferation of wild-type T cells.

Collectively, these data indicate that when Ikaros levels are reduced, T cells become less sensitive to inhibitors of proximal and distal components of the PKC, PI-3 kinase and PLCγ signaling pathways. This suggests that the phenotype manifested in Ikaros-mutant T cells is due to a molecular defect downstream of these pathways. As Ikaros activity is reduced, a T cell becomes less signal-dependent for activation. Ikaros protein may thus serve as a major nuclear target for the multiple signaling pathways downstream of TCR and IL-2R which modulate its activity to allow for regulated cell cycle progression.

Changes in Nuclear Localization of Ikaros in Activated T Cells

Taken together, these activation studies suggest that Ikaros activity may change during the T cell cycle. Such changes do not occur at the level of expression, as *Ikaros* mRNA decreases slightly and protein levels remain unaffected (Figures 2A and 2B). However, changes in the nuclear localization of Ikaros protein were detected. In the nuclei of primary resting T cells, Ikaros protein was detected in a diffuse reticular pattern that was excluded from nucleoli, as well as in a number of discrete foci (Figure 4A). Upon T cell activation, the nuclear pattern of Ikaros staining changed markedly to reveal Ikaros protein concentrated in intensely staining toroidal structures (Figure 4B).

To gain insight into how changes in the Ikaros protein complex correlate with cell cycle transitions, a more detailed analysis was undertaken. Purified splenic T cells from both wild-type and DN$^{+/-}$ animals were activated for different lengths of time and pulsed with BrdU prior to harvesting to reveal cells in S phase. Resting wild-type and DN$^{+/-}$ T cells displayed a similar reticular/punctate pattern of Ikaros staining and were negative for BrdU (Figures 5A and 5B). By 16 hr of activation, most of the wild-type T cells stained negative for BrdU and exhibited a small increase in nuclear size, indicating they were in G1 (Figure 5A). Small Ikaros toroids were detected at this stage. By 24 hr of stimulation cells were in mid-late G1, as indicated by a 2- to 3-fold increase in nuclear size and lack of BrdU staining. At this stage Ikaros protein was detected in large toroids (Figure 5A). In contrast to wild-type T cells, a significant proportion of Ikaros DN$^{+/-}$ T cells stained positive for BrdU by 16 hr, demonstrating their faster progression to S and confirming the results shown in Figure 1C.

By 38–44 hr of TCR stimulation wild-type T cells were

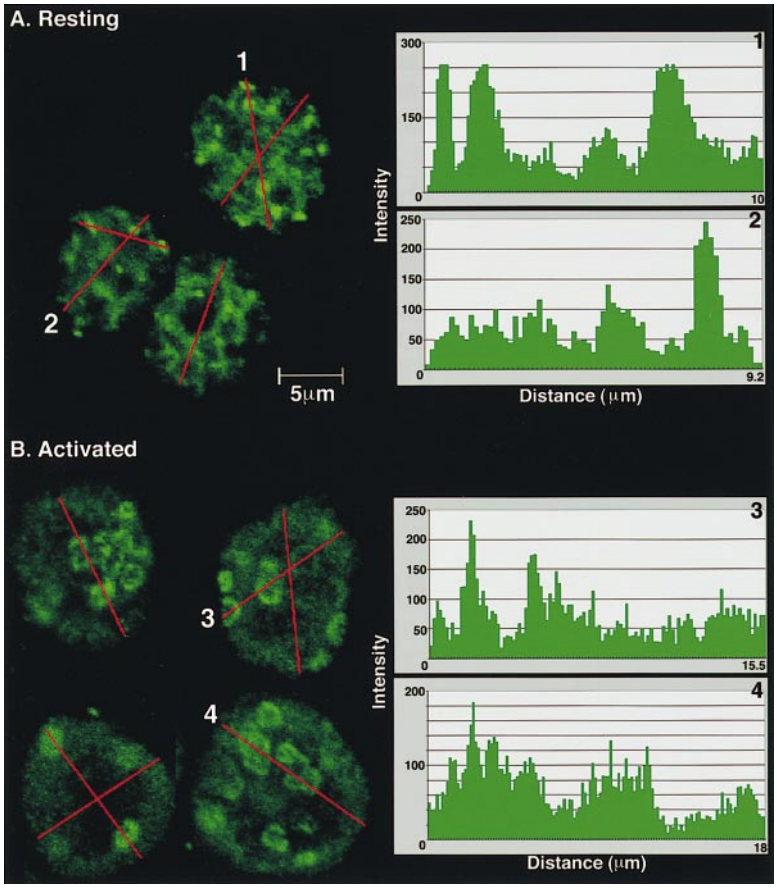


Figure 4. Upon T Cell Activation, Ikaros Is Incorporated into a Higher-Order Nuclear Structure

(A) In purified resting T cells, Ikaros protein is present throughout the nucleus in a diffuse-reticular pattern of staining and is also concentrated in small discrete foci. The ratio of intensity of Ikaros staining in dots versus diffuse areas is 2–5. Red lines demarcate measurements of staining intensity across the nucleus. Two representative histograms (1 and 2) are shown.

(B) Upon T cell activation Ikaros localizes in macromolecular toroidal structures. 3D reconstruction of these structures shows that they are actually cylinders that range from 2–3.5 μM in length and 1.5–2.54 μM in diameter. Two representative intensity histograms (3 and 4) across the nucleus of an activated T cell are shown. The y-axis indicates fluorescence intensity, the x-axis indicates distance across the nucleus (in μm). Note that upon T cell activation the intensity of the diffuse Ikaros nuclear staining is decreased relative to the resting state. The same PMT was used to collect images for resting and activated T cells and background staining was subtracted prior to image collection.

in S phase, as revealed by positive staining for BrdU (Figure 5A), and displayed Ikaros in toroids. The BrdU staining patterns of these cells indicated that they were at various stages of S phase, ranging from early (diffuse/punctate pattern) to mid (perinuclear) to late (toroids) (Nakayasu and Berezney, 1989; O'Keefe et al., 1992). Irrespective of the stage of S phase, Ikaros protein displayed a toroidal pattern. In $DN^{+/-}$ T cells, Ikaros toroids were less striking, and a more intense diffuse pattern of staining was observed (Figure 5B). Thus, there is an inverse correlation between the proliferative capacity of Ikaros $DN^{+/-}$ T cells and the formation of Ikaros toroids, suggesting that these higher-order nuclear structures may negatively regulate the G1-S progression.

To delineate the signaling events required for the formation of Ikaros toroids we used inhibitors of PKC, PI-3, p70 S6 and tyrosine kinases, which are critical components of the TCR and IL-2R signaling pathways. PP1, staurosporine and PD98059 (which target TCR signaling pathways) reduced both the number of G1 cells with Ikaros toroids and the number of S phase cells (data not shown). However, rapamycin and wortmannin (which target the IL-2R signaling pathway as inhibitors of the p70 S6 kinase) effectively blocked entry into S yet had a relatively small effect in reducing the number of G1 cells with Ikaros toroids (data not shown). These data suggest that the formation of Ikaros toroids lies downstream of TCR signaling and occurs prior to the events required for G1 to S transitions triggered by IL-2R signaling.

Ikaros Toroids Colocalize with Sites of DNA Replication

The Ikaros toroids that form in activated T cells are reminiscent of the staining patterns of DNA replication foci forming at late S (as revealed by BrdU labeling) and of proteins which associate with DNA replication factories, such as methyltransferase, PCNA, Cyclin A and cdk2 (Leonhardt et al., 1992; Cardoso et al., 1993; Hozák and Cook, 1994). We, therefore, examined the possibility that Ikaros proteins colocalize with DNA replication foci and factors.

In activated wild-type T cells, Ikaros toroids colocalized with a subpopulation of BrdU-labeled DNA replication clusters (Figures 6A–6C). The staining pattern of the DNA replication foci with which Ikaros colocalized was indicative of regions of heterochromatin which replicate at the mid-late stages of S phase, such as centromeric heterochromatin and heterochromatin at the interior part of the nucleus (Nakayasu and Berezney, 1989; O'Keefe et al., 1992). Ikaros toroids localize in the periphery of heterochromatin, as visualized by Hoechst staining; however, Ikaros ''speckles'' in resting cells are mostly excluded from these sites (data not shown). We also tested whether Ikaros colocalized with proteins such as methyltransferase, which acts on replicating DNA.

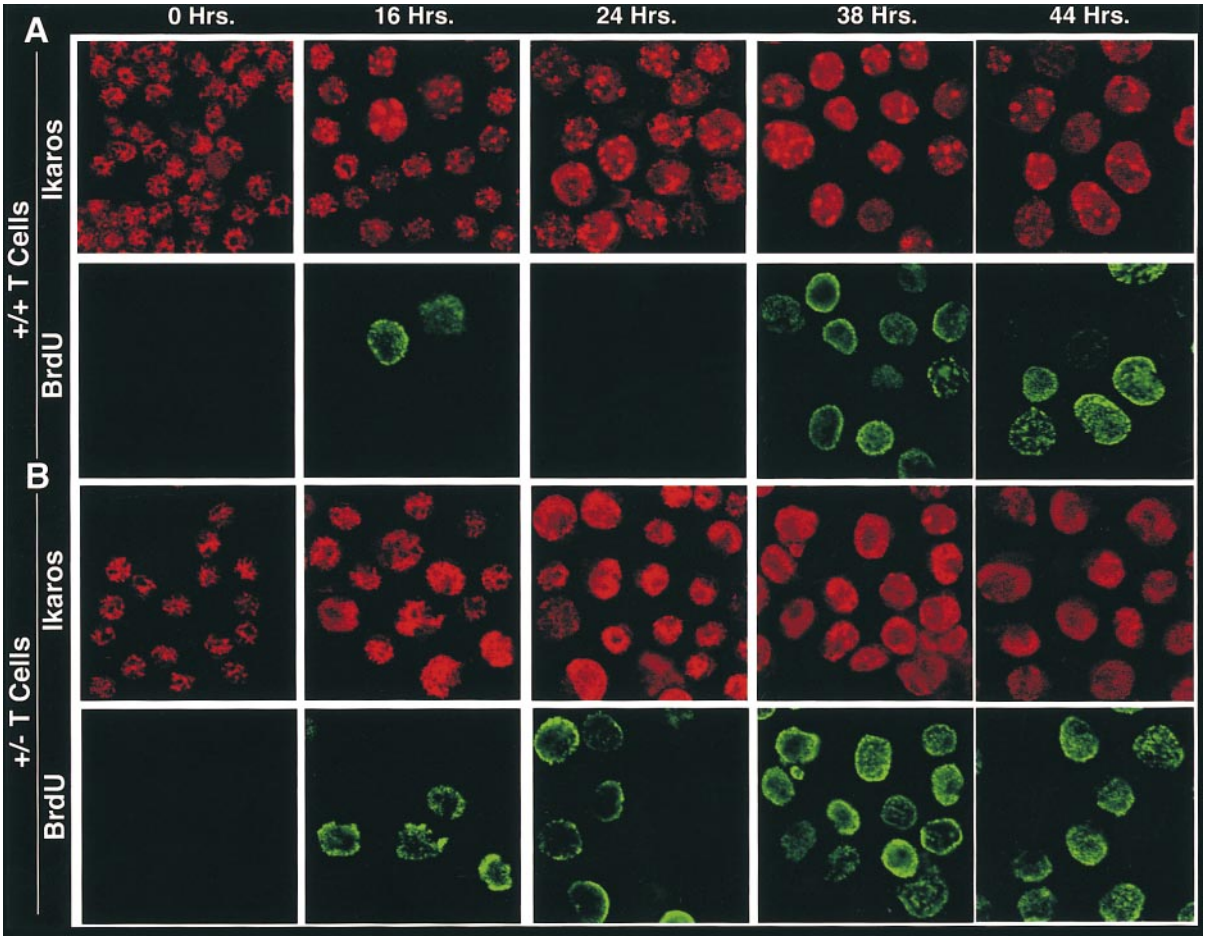


Figure 5. Changes in Nuclear Ikaros Structures in Wild-Type and Dominant-Negative Ikaros-Mutant T Cells upon Activation

Purified splenic T cells from +/+ mice (A) and from Ikaros $DN^{+/-}$ mice (B) were activated with 20 μg/ml plate bound anti-CD3ε and pulsed with BrdU for 10 min prior to harvesting at the indicated time points. Cells were stained with antibodies against BrdU (shown in green) to identify cells in S phase and Ikaros (shown in red). Both resting +/+ and $DN^{+/-}$ T cells show a punctate pattern of Ikaros staining over a diffuse reticular pattern (0 hr). By 24 hr after TCR stimulation, Ikaros is visualized in toroids in of +/+ T cells and very few cells have incorporated BrdU, indicating that they are in G1. By 38 hr, the majority of +/+ T cells are in S phase, and Ikaros is predominantly seen in toroids. In activated $DN^{+/-}$ T cells, formation of Ikaros toroids is not seen clearly. Moreover, significant numbers of $DN^{+/-}$ cells label with BrdU as early as 16 hr after activation, indicating shortening of G1 phase.

Methyltransferase has been shown to form toroids which colocalize with replicating heterochromatin in mid-to-late S phase (Leonhardt et al., 1992). Significantly, the colocalization of Ikaros and methyltransferase was 100% (Figures 6D–6F). Colocalization with cyclin A, which associates with DNA replication mega-complexes, was also detected (Figures 6G–6I). Although Ikaros is detected in the nucleus both in resting T cells and in G1 through S phase of the cell cycle, it is not detected on condensed chromosomes during early-mid M phase. Specifically, Ikaros is not detected in prophase, metaphase and anaphase, but it rapidly reappears in a punctate-reticular pattern in dividing nuclei at telophase and prior to cytokinesis (Figures 6J and 6K).

Collectively, the colocalization of activation-induced higher-order Ikaros structures with replication-associated enzymes, like methyltransferase and cyclin A, and a subset of replication foci suggests a possible role for Ikaros in DNA replication.

Chromosomal Aberrations among Cycling T Cells with Reduced Levels of Ikaros

Given a potential role for Ikaros in DNA replication and chromosome propagation, we examined the karyotypes of cycling wild-type and Ikaros $DN^{+/-}$, $null^{+/-}$ and $null^{-/-}$ T cells. Chromosome spreads of T cells arrested in metaphase after 2–3 cell cycles were examined (Figure 7A). Polyploidy counts on 100 metaphases per genotype showed insignificant differences between Ikaros-mutant and wild-type T cells, with 2% of wild-type and 1–6% of Ikaros-mutant metaphases being tetraploid. For each genotype, exact chromosome counts were also obtained on 30–40 metaphases. The number of monosomies or trisomies (39 or 41 chromosomes) was significantly higher among Ikaros-mutant relative to wild-type T cells (Figure 7B, 5% for wild type, 40% for $null^{+/-}$, 30% for $DN^{+/-}$ and 17% for $null^{-/-}$).

Changes in the size of one of the members of chromosome 1 pair were consistently seen in the majority of

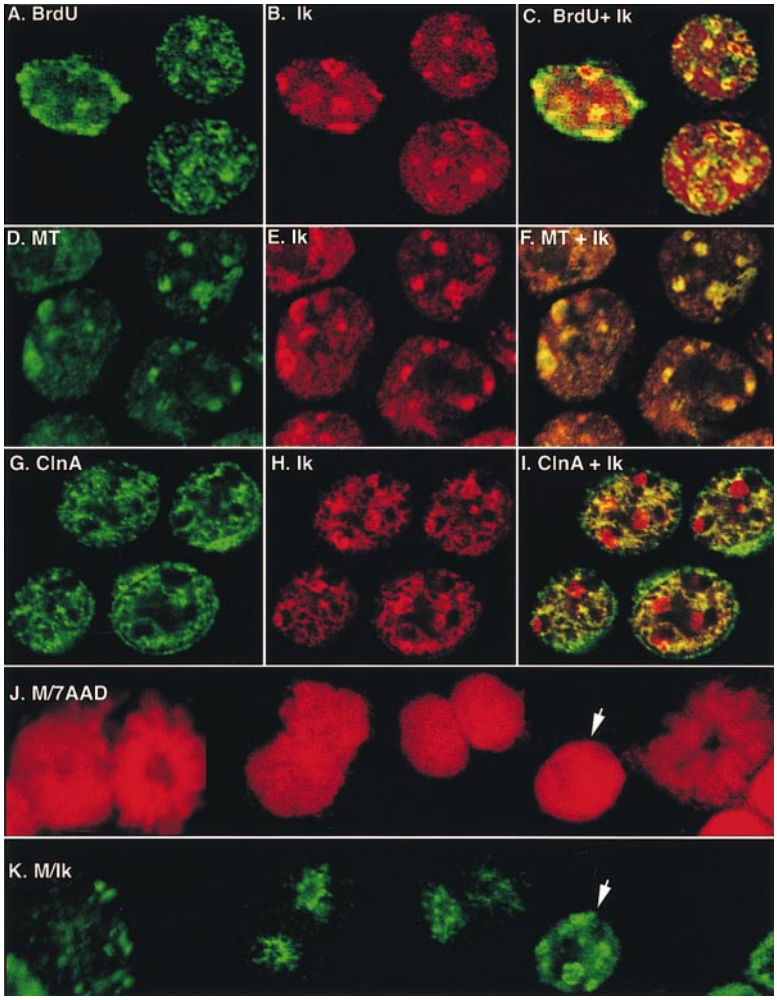


Figure 6. In activated T cells Ikaros colocalizes with methyltransferase, Cyclin A and a subset of DNA replication foci

BrdU-labeled T cells were costained for BrdU (A), methyltransferase (D) or Cyclin A (G) and Ikaros (B, E, H).

Superimposition of the two stainings reveals a number of cells where Ikaros colocalizes with BrdU and Cyclin A (seen as yellow, C and I). Colocalization between Ikaros and methyltransferase (seen as yellow, F) is 100%. The discrete pattern of BrdU foci in these cells demarcates replication of heterochromatic regions which are distributed from the nuclear periphery to the interior part of the nucleus and occurs during mid-late S-phase. DNA staining with 7-aminoactinomycin D (7AAD) (shown in red, J) reveals that Ikaros protein (shown in green, K) is not detected on mitotic chromosomes in metaphase and anaphase. However, Ikaros is detected in telophase and in cells undergoing cytokinesis. An arrow indicates the only cell in this field which is not undergoing mitosis or cytokinesis and which has Ikaros in toroidal structures.

Ikaros-mutant metaphases (Figures 7B and 7C). The most commonly affected region was between bands A1-A4 of chromosome 1 (Figure 7B). In addition, extrachromosomal fragments, classified as minutes and usually associated with gene amplification events (Sen et al., 1989), were seen in a number of Ikaros-mutant metaphase spreads (Figure 7D). Changes in band size of other chromosomes and chromatid breaks were detected at a lower frequency.

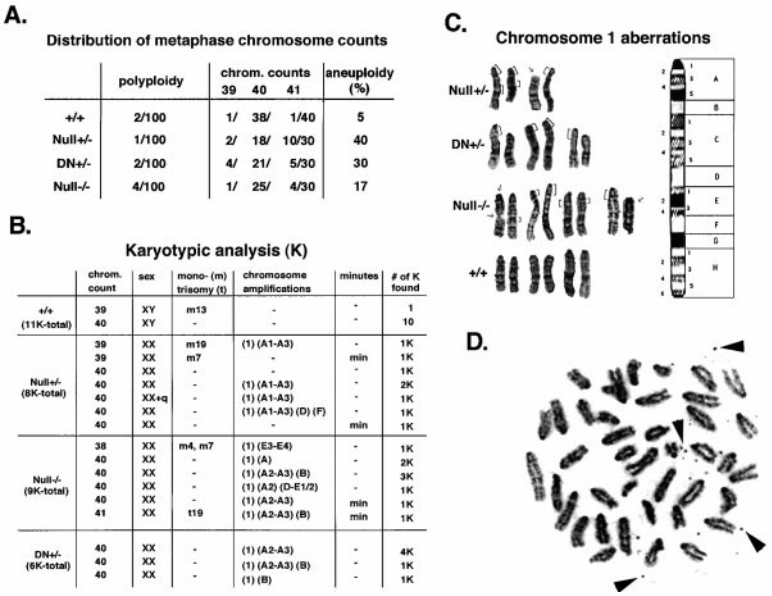
Thus, a reduction in Ikaros activity in primary T cells causes the frequent occurrence of chromosome aberrations. This chromosome infidelity detected in vitro among proliferating Ikaros-mutant T cells suggests a mechanism by which these cells transform to a malignant state in vivo in the process of providing immunity (Winandy et al., 1995).

Discussion

Our current studies reveal that TCR and IL-2R signaling thresholds which control entry and progression through the T cell cycle are set by Ikaros proteins. Upon TCR signaling, Ikaros proteins form a higher-order chromatin structure which we propose serves as a critical regulator of T cell activation.

The activation of a resting primary T cell is divided into two steps. The first step is described as the early G1 "competency" phase, which requires a critical number of TCR-complex engagement events to signal changes in the expression of growth factors and receptors (i.e., IL-2 and IL-2R). The second step, known as "progression," occurs in a growth factor (e.g., IL-2)-dependent fashion and promotes progression through a "restriction point" into S phase by inducing changes in cell cycle regulators such as cyclins, cyclin dependent kinases and inhibitors of these kinases (Turner, 1993; Hunter and Pines, 1994; Nourse et al., 1994; Cantrell, 1996; Kwon et al., 1997). Changes in these cell cycle regulators ultimately affect the activity of the DNA replication machinery.

Both steps in T cell activation are deregulated when Ikaros levels are reduced. In Ikaros-mutant T cells, "competency" occurs in response to a reduced number of TCR signaling events and at low cell density, i.e., conditions which are insufficient to promote the G0-G1 transition of T cells with normal levels of Ikaros activity. Ikaros-mutant T cells hyperrespond to IL-2 and enter S phase at a faster rate relative to wild-type T cells. These effects are not due to an increase in IL-2 production or to significant changes in expression of IL-2R. Several



A.

Distribution of metaphase chromosome counts

	polyploidy	chrom. counts 39	40	41	aneuploidy (%)
+/+	2/100	1/	38/	1/40	5
Null+/-	1/100	2/	18/	10/30	40
DN+/-	2/100	4/	21/	5/30	30
Null-/-	4/100	1/	25/	4/30	17

B.

Karyotypic analysis (K)

	chrom. count	sex	mono- (m) trisomy (t)	chromosome amplifications	minutes	# of K found
+/+ (11K-total)	39	XY	m13	-	-	1
	40	XY	-	-	-	10
Null+/- (8K-total)	39	XX	m19	(1) (A1-A3)	-	1K
	39	XX	m7	-	min	1K
	40	XX	-	-	-	1K
	40	XX	-	(1) (A1-A3)	-	2K
	40	XX+q	-	(1) (A1-A3)	-	1K
	40	XX	-	(1) (A1-A3) (D) (F)	-	1K
	40	XX	-	-	min	1K
Null-/- (9K-total)	38	XX	m4, m7	(1) (E3-E4)	-	1K
	40	XX	-	(1) (A)	-	2K
	40	XX	-	(1) (A2-A3) (B)	-	3K
	40	XX	-	(1) (A2) (D-E1/2)	-	1K
	40	XX	-	(1) (A2-A3)	min	1K
	41	XX	t19	(1) (A2-A3) (B)	min	1K
DN+/- (6K-total)	40	XX	-	(1) (A2-A3)	-	4K
	40	XX	-	(1) (A2-A3) (B)	-	1K
	40	XX	-	(1) (B)	-	1K

C.

Chromosome 1 aberrations

D.

Figure 7. Chromosome Aberrations Occur Rapidly in Cycling Ikaros-Mutant T Cells

(A) Ploidy counts of +/+ vs Ikaros-mutant T cells are displayed as the number of polyploid cells per 100 metaphases (polyploidy), exact chromosome counts for 30–40 metaphases (chrom. counts) and the percent aneuploidy.

(B) Representative cytogenetic analysis of one of four studies carried out with purified splenic T cells. A close examination of chromosomes was performed on 6–11 metaphases with a diploid or aneuploid number of chromosomes.

(C) Pairs of chromosome 1 from wild-type and Ikaros-mutant metaphases with a diploid number of chromosomes. The typical banding pattern of chromosome 1 is shown on the right. Changes in regions along this chromosome are shown with brackets. These result in a difference in the size of chromosome 1 frequently seen among Ikaros-mutant metaphases. Arrows indicate deletions proximal to the centromere and chromatid breakpoints.

(D) Ikaros-mutant chromosomal spreads frequently show the presence of minute chromosomes (arrows).

key nuclear effectors, cyclin D, E, and A, cdk2, and the cdk inhibitors p27, p19 and p21, which coordinate entry and progression through the cell cycle, are also unaffected. However, it cannot be ruled out that activity of some other cell cycle regulator is altered.

Lack of Ikaros may affect the signaling network downstream of TCR and IL-2R, yet attenuators of TCR signaling, like CTLA-4 and its downstream effector SHP-1, are expressed normally in Ikaros-mutant T cells. Tyrosine phosphorylation levels in resting and activated T cells are also similar, indicating that, in the absence of Ikaros, signaling pathways are not constitutively active or hyperreactive. Unexpectedly, a number of pharmacological inhibitors of proximal and distal components of the PKC, PI-3 kinase and calcineurin signaling cascades are less effective in blocking proliferation of Ikaros-mutant T cells, suggesting that the defect lies downstream of these points. Since signaling through all three pathways is affected, Ikaros is implicated as a "molecular point of convergence."

The TCR signaling cascades may regulate entry into the T cell cycle by altering the activity of Ikaros proteins. In support of this hypothesis, TCR signaling modulates the nuclear pattern of Ikaros protein. Inhibitors of TCR signaling cascades reduce the number of G1 cells with Ikaros toroids. In quiescent T cells, Ikaros is detected in a nuclear reticular/punctate pattern mostly not associated with heterochromatin. Upon TCR-mediated T cell activation, a distinctive network of 8–16 Ikaros toroids is discerned which occupies a major part of the nuclear volume. Reduction in Ikaros toroids, observed in T cells with mutations in the Ikaros gene, correlates with lower TCR signaling thresholds and accelerated entry into S phase. Thus, formation of the Ikaros higher-order chromatin structures may be critical for the regulated progression of a T cell through the cell cycle.

The localization of Ikaros proteins in these "cup-like" structures in G1 has been previously reported in B cell lines (Brown et al., 1997), and is consistent with our findings. These Ikaros structures were shown to be in close apposition to centromeric heterochromatin (Brown et al., 1997). In addition, our experiments with BrdU-labeled primary cells demonstrate that the Ikaros toroids, which form in early-mid G1, are maintained through S phase and colocalize with a subset of DNA replication foci whose staining pattern is indicative of regions of late-replicating heterochromatin (O'Keefe et al., 1992). In this higher-order chromatin structure, Ikaros shows coincidental staining with methyltransferase and Cyclin A, which associate with and regulate the DNA replication megacomplex (Leonhardt et al., 1992; Cardoso et al., 1993; Hozák and Cook, 1994). Colocalization of Ikaros in a chromatin network with DNA replication foci and enzymes suggests a potential role in DNA replication which may be to form chromatin boundaries that regulate DNA accessibility to the transcriptional and DNA replication machineries (J. Kim, unpublished data; J. Koipally, unpublished data).

Consistent with this hypothesis, changes in chromosome bands are detected in primary Ikaros-mutant T cells after limited in vitro proliferation. An increase in the size of chromosome 1 is consistently seen among the majority of Ikaros mutant metaphases. Changes in the banding pattern of other chromosomes is detected at a lower frequency. An increase in the size of chromosome bands and extrachromosomal fragments frequently seen in Ikaros-mutant metaphases can result from DNA amplification (Sen et al., 1989). Thus, a reduction of Ikaros activity facilitates T cell activation and may provide the first step towards transformation. Additional defects caused by unfaithful propagation of genetic material may contribute to the rapid development of T cell neoplasias in Ikaros-mutant mice and suggest a novel function for Ikaros as a tumor suppressor.

Cell division can also influence the state of differentiation by giving rise to daughter cells which are distinct from the mother cell. An increasing body of work indicates that maintenance of old or induction of new gene

programs in daughter cells is coupled to processes which occur either during replication or immediately after (Almouzni and Wolffe, 1993; Brandeis et al., 1993; Jeppesen, 1997; Araujo et al., 1998). In this light, it is interesting that Ikaros colocalizes with methyltransferase, which serves to direct replication-coupled DNA methylation and may serve to alter gene expression in the daughter cell, resulting in a more differentiated state. Studies on the chromatin-based role of Ikaros in the stable propagation of genetic material and its potential link to gene expression may provide the molecular basis for the control of lymphocyte differentiation and proliferation.

Experimental Procedures

T Cell Purification

All mice used for these studies were C57Bl/6 × 129SV. Lymphoid organs were removed aseptically from age-matched (4–6 weeks) wild-type, Ikaros null$^{+/-}$, and DN$^{+/-}$ mice and single-cell suspensions were made as previously described (Georgopoulos et al., 1994). T cell purification was performed using the VarioMacs (Miltenyi Biotech) (80%–90% purity) or the high speed MoFlo flow cytometer (Cytomation, Inc.) (95%–98% purity). TCR expression was determined with H57-597 monoclonal antibody (Pharmingen).

T Cell Proliferative Responses

Flat-bottomed 96-well plates were coated with anti-CD3ε (145-2C11, Pharmingen). Cells were plated in triplicate in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 μM β-mercaptoethanol and 50 μg/ml gentamicin. Cells were pulsed for 6–12 hr with ^{3}H-methyl thymidine (2 μCi/well) at various time points after culturing, harvested (Harvester 96/TomTec) and counted (Betaplate Counter/Wallac).

Analysis of Cytokine Production

Splenocytes from +/+, Null$^{+/-}$, and DN$^{+/-}$ mice were activated with 25 μg/ml plate-bound anti-CD3ε and 2 μg/ml soluble anti-CD28 (Pharmingen) for 5 hr prior to analysis. Brefeldin A was added to the cultures 2 hr after activation. Cells were stained with antibodies against CD4 and CD8, fixed and permeabilized (Cytofix/Cytoperm Plus/Pharmingen). Staining with monoclonal antibodies against IL-2, IL-4 and IFN-γ was then performed.

Metaphase Counts, Chromosome Spreads, and Karyotyping

Purified splenic T cells were cultured for 72 hr in the presence of plate-bound anti-CD3ε and then treated with colchicine to arrest proliferating cells in metaphase. Karyotypes were prepared from Giemsa stained chromosome spreads from wild-type and Ikaros-mutant T cells. Part of these studies were performed by Dr. B. Hukku at the Cell Culture Laboratory, Children's Hospital of Michigan, Detroit, MI.

Northern Analysis

RNA was prepared using standard methods (Chomczynski and Sacchi, 1987). Probes used for analysis were IL-2Rα, GAPDH, Ikaros, p27 (gift of T. Halazonetis). Quantitations were performed with a PhosphorImager (Molecular Dynamics) and ImageQuant software.

Western Blot Analysis

Whole cell extracts were prepared in 10 mM Tris (pH 7.5), 1% SDS, 1 mM PMSF, and 1 mM Na_3VO_4. Proteins were separated by 12% SDS-PAGE and transferred to Immobilon-P. Blots were incubated with specific antibodies at 4°C overnight in PBS/5% milk/0.1% TWEEN (pH 7.4), followed by HRP-secondary antibody and visualized with ECL (Amersham). Polyclonal antibodies to p27, p19, cyclins D (cross-reactive with D1, D2 and D3), E and A, and cdk2 (Santa Cruz, CA), Ikaros and p21 (gift of E. Calautti) were used.

Immunofluorescence

Purified splenic T cells were prepared and stained as previously described (Wang et al., 1988). To visualize Ikaros and cyclin A, cells were permeabilized for 5 min in PBS/0.5% Triton, fixed for 20 min in 4% paraformaldehyde and stained as described. Anti-methyltransferase (anti-pATH52) was a gift of T. Bestor.

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Note Added in Proof

The data referred to throughout as ''J. Kim, unpublished data'' are now in press: Kim, J., Sif, S., Jones, B., Jackson, A., Koipally, J., Heller, E., Winandy, S., Viel, A., Sawyer, A., Ikeda, T., Kingston, R., and Georgopoulos, K. (1999). Ikaros DNA-binding proteins direct formation of chromatin remodeling complexes in lymphocytes. Immunity *10*, 345–355.